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13. ABSTRACT ***(Maximum 200 Words)***

Disruption of the TGF-β signaling pathway in the mammary stroma results in aberrant ductal morphogenesis. Our laboratory has developed transgenic mice that express a Zn^{2+} inducible, kinase-defective dominant-negative TGF-β type II receptor in the mammary stroma (MTR28). Female mice expressing the dominant-negative receptor demonstrated increased lateral branching, suggesting TGF-β signaling in the stroma has important effects on epithelial morphogenesis. Recent evidence has accumulated implicating a role for the stroma in regulating tumor formation. To determine if loss of responsiveness to TGF-β in the stroma effects tumor development transgenic and wild type mice were given pituitary isografts followed by zinc water and either left untreated (control) or treated with 7,12-dimethylbenz (a) anthracene (DMBA). Several tumors developed in the wild type group on a full regiment (pituitary isograft, zinc and DMBA) while only one tumor has arisen in the control group. To date, only two tumors have arisen in the transgenic mice on a full regiment. To identify genes regulated by TGF-β in the mammary stroma, filter and gene chip microarrays were screened, also subtracted cDNA libraries produced by suppression subtractive hybridization (SSH) were arrayed ont glass slides. The filter based and commercial gene chip microarrays identified genes expressed in the mammary stroma and directly regulated by TGF-β. The genes were verified by Northern analysis and *in situ* hybridization. The expression of several categories of genes were affected by loss of TGF-β signaling as determined by sequence analysis of the SSH libraries.

14. SUBJECT TERMS
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Table of Contents

The transforming growth factor betas (TGF-β1, -β2, and -β3) are members of a family of peptide growth factors that include inhibins, bone morphogenic proteins (BMPs) and growth and differentiation factors (GDFs) (1-3). Activation of the TGF-β receptor results in Smad activation and ultimately to changes in gene expression. TGF-βs have long been associated with diverse cellular processes including but not limited to growth arrest in epithelial cells, cell proliferation and differentiation of mesenchymal cells during development, cell migration in wound healing, extracellular matrix production and immunosuppression. The TGF-β's and their receptors are expressed throughout the mammary gland where it has a negative effect on the growth of the ductal epithelium and lobuloalveolar development depending on the timing of TGF-β expression. For example, mouse mammary tumor virus (MMTV) driving expression of active TGF-β1 produced hypoplastic growth of the ductal epithelium (4). In contrast, expression of active TGF-β1 during pregnancy by the whey acidic protein (WAP) promoter resulted in decreased lobuloalveolar development and failure of terminal differentiation (5). The reciprocal experiments, expression of a kinase-deficient dominant-negative TGF-β type II receptor (DNIIR) in the mammary epithelium and in stromal fibroblasts resulted in precocious lobuloalveolar development and increased lateral branching, respectively, compared to wild type littermates (6,7). These data confirm the requirement for the mammary stroma and TGF-β for proper ductal morphogenesis. In mice, several studies support TGF-β signaling as a tumor suppressor for the mammary gland. Expression of transforming growth factor-alpha (TGF-α) in mammary epithelial cells resulted in mammary ductal hyperplasia that progressed to carcinomas (8). Co-expression of TGF-β1 in the mammary epithelium significantly reduced the number of tumors induced by the TGF-α transgene (9). In addition, TGF-β expression also inhibited tumor formation when mice expressing only the TGF-β transgene were challenged with 7,12-dimethybenz (a) anthracene (DMBA) (9). In a separate experiment, DMBA treatment of mice expressing the DNIIR transgene in the mammary epithelium, eliminating TGF-β signaling, increased the incidence and number of tumors (10). Furthermore, a separate mouse line expressing DNIIR in the epithelium demonstrated mammary tumors stochastically, without carcinogen treatment, after a long latency period (2 1/2 years) but at a moderately high frequency (~45%) (H. L. Moses, personal communication) supporting a tumor suppressor function for TGF-β in the mammary gland. Therefore, the purpose of this proposal is to test the overall hypothesis that development of mammary tumors will be altered in the absence of TGF-β receptor signaling in the mammary stroma. It is also hypothesized that there are changes in gene expression resulting from the lack of TGF-β signaling in the mammary stroma that will ultimately influence tumor development. To test these hypotheses Serra and colleagues have engineered a mouse transgenic for a kinase-defective dominant-negative TGF-β type II receptor driven by a metallothionine promoter (MTR28), which allows for regulation of the transcript by heavy metals (e.g. zinc sulfate) (7). Assessing what role, if any, the lack of TGF-β receptor signaling in the mammary stroma affects mammary neoplasia will help to identify novel diagnostic or therapeutic strategies heretofore unexplored.

Previously, Joseph, et al., (1999) had shown that the loss of responsiveness to TGF-β signaling in the mammary stromal fibroblasts resulted in increased lateral branching of the mammary ductal epithelium. Moreover, it has been shown that the mammary stroma may contribute to the generation of experimentally induced mammary tumors (11). Therefore, the purpose of this proposal was to test the hypothesis that development of mammary tumors will be altered in the absence of TGF-β receptor signaling in the mammary stroma. To that end, tumor development was compared in wild type and zinc-inducible kinase-deficient dominant-negative TGF-β type II receptor (MTR28) transgenic mice (7). As stated in Task 1 of the Statement of Work (S.O.W.), four groups of mice were established with approximately 25 animals per group. The groups consisted of wild type mice given pituitary isografts and zinc-water (Group 1), wild type mice with pituitary isografts, zinc-water and carcinogen (Group 2), MTR28 transgenic mice with pituitary isografts and zinc-water (Group 3) and MTR28 transgenics with pituitary isografts, zinc-water and carcinogen (Group 4). When female mice were 5 weeks of age pituitaries from sibling male mice were removed and implanted under the kidney capsule of the recipient female. The mice were placed on zinc-water at 6 weeks of age to induce the transgene or to control for

the effects of zinc, if any, on gene expression. The mice were then either treated weekly for four weeks with 1mg of 7,12-dimethybenz (a) anthracene (DMBA) beginning at 8 weeks of age or not treated with the carcinogen. To date 52 wild type mice have received a pituitary isograft under the kidney capsule and are being treated continuously with $ZnSO_4$ in the drinking water. These mice do not carry the DNIIR transgene; therefore they do not express the dominant-negative form of the TGF-β type II receptor and have a functional TGF-β response. Twenty-six mice are currently approaching 1 year of pituitary and zinc treatments without carcinogen and only one tumor has arisen in these mice. The second group with 26 wild type mice was treated with DMBA and 7 tumors have developed in these mice with an average of 1.5 tumors per mouse and with an average latency period of approximately 2.5 months. Three mice were sacrificed when the mice were moribund but without palpable mammary tumors. At least one of these mice was identified as having leukemia at the time of necropsy. This number of moribund mice was expected as DMBA induces leukemia and lymphoma in addition to mammary carcinomas. All of the tumors from the wild type mice displayed areas of swirling keratinazation indicative of adenoacanthoma (Fig.1). The wild type mice with pituitary isografts, on zinc-water and dosed with carcinogen have had the treatment for an average of 7.3 months out of a study period of one year. Twenty-seven MTR28 mice have received the pituitary isograft, are on zinc-water and have been fully dosed with carcinogen and to date only two tumors have developed. One tumor displayed an adenoacanthoma phenotype similar to the wild type mice, while the other displayed an adenocarcinoma-like phenotype (Fig. 2A and B respectively). The average time period for carcinogen treatment for the MTR28 mice has been 3 months. Tumors usually begin to arise between 4 and 6 months after the last dose of carcinogen. Therefore, more tumors should arise over the passage of time in these mice. The last group of mice, MTR28 transgenics with pituitary isografts and zinc-water only, are now being collected. Prior to the beginning of the current study the MTR28 transgenic line was reduced to only a few animals and breeding to expand the numbers required for the study was slow. This was due mainly to reduced litter size in the MTR28 breeding population. There are now 7 breeding pairs that are producing normal (8-10 pups) size litters.

In order to identify genes whose expression was altered by the loss of TGF-β responsiveness in the mammary stroma several different microarrays were screened. As discussed above, in response to zinc the MTR28 mice lack a functional TGF-β response in the mammary stromal fibroblasts (7). In contrast, mammary fibroblasts isolated from non-transgenic mice and grown in culture retain a functional TGF-β response. By comparing the expression profiles of genes in the transgenic mammary gland to that from the cells *in vitro*, it is possible to find genes that are directly regulated by TGF-β in mammary stromal fibroblasts. RNA was isolated from the mammary glands of wild type and MTR28 transgenic mice treated with zinc sulfate for 1 week and from fibroblasts in culture treated and not treated with TGF-β1. Initially, filter based gene array was done to test the validity of the screening process. The RNA isolated was converted to cDNA using standard techniques and radioactively labeled for filter based gene array analysis. Filter based gene array was done using the mouse 1.2 release from Clontech (CA) which contained a combination of 1,200 known genes and ESTs. The filters were first screened using RNA isolated from mammary glands from wild type and MTR28 transgenic mice. The filters were subsequently re-hybridized with RNA isolated from primary mammary fibroblasts either treated or untreated with TGF-β (these cells were isolated from normal mice and contain a functional TGF-β response). This screening protocol resulted in the identification of genes expressed in the mammary stoma and regulated by TGF-β. For example, G-protein coupled receptor-27 (GPCR27) was found to be down-regulated in the mammary gland from transgenic mice relative to the wild type and up-regulated in the primary mammary stromal fibroblasts treated with TGF-β (Fig 3). The expression profile in the transgenic and wild type mammary glands and in the fibroblasts treated and untreated with TGF-β was verified by Northern analysis (Fig. 3A). GPCR27 expression was further demonstrated in the cleared fat pad and in the mammary gland of non-parous mice at different ages while expression levels dropped during pregnancy and were virtually non-existent during lactation (Fig. 3B). Localization of the transcript was verified to the mammary stroma via *in situ* hybridization; however,

some localization could be detected in the epithelium (Fig. 3C). This result was not unexpected as a very low level of expression was detected in mammary epithelium by Northern analysis (Fig. 3B). Using the strategy described above 8 genes were identified whose expression met the criteria (up in one and down in the other) but only 2 genes (GPCR27 and Wnt 11) were verified by Northern analysis (Fig. 3 and data not shown). These data confirmed that genes could be isolated from the mammary stroma and are directly regulated by TGF-β.

In addition to screening the filter based gene array a slide based "gene chip" was also analyzed for altered gene expression in the mammary glands and isolated mammary fibroblasts. The slide based gene chip microarray from Incyte (company location here) containing 8,000 known genes and ESTs was hybridized with RNA isolated from mammary glands from wild type and transgenic mice treated with zinc sulfate for 1 week as well as from primary mammary stromal fibroblasts treated or un-treated with TGF-β (Fig 4). Labeling of the RNA and hybridization of the chips were done in the University of Cincinnati's microarray core facility. The data generated were analyzed using the GeneSpring software. Scatter plot analysis of the arrays resulted in the identification of 48 known genes and ESTs that were up-regulated in the transgenic mammary glands relative to the wild type (Fig 4A and B). By contrast, only 15 genes and ESTs were down-regulated in this group (Fig 3A and B). Hybridization of the microarrays with RNA isolated from primary mammary fibroblasts treated and untreated with TGF-β resulted in 24 known genes and ESTs identified as up-regulated and 30 genes and ESTs being down-regulated (Fig 4C and D). The largest category of up-regulated genes in the transgenic mammary glands relative to wild type were those involved in metabolism (12 genes) with lipid metabolism (8 genes) and the immune response (7 genes) the categories with the next highest number of genes. Genes involved in protein degradation (4 genes) was the largest category in the fibroblasts stimulated with TGF-β. The genes down-regulated in the transgenic mammary glands and in the mammary fibroblasts were varied and included proteins associated with the membrane and immune response among others. Several genes are currently being evaluated for verification by Northern blot analysis or RT-PCR analysis or both.

In task two of the S.O.W. it was hypothesized that changes in gene expression that result from the lack of TGF-β signaling in the mammary stroma may ultimately influence tumor development. To isolate genes whose expression profiles have changed as a result of lack of TGF-β signaling in the mammary stroma tissue specific "gene chips" were created. Suppression-subtraction hybridization (SSH) was done using PCR-select cDNA subtraction from Clontech as per the manufacturer's instructions to select for genes up-regulated and down-regulated in the mammary glands isolated from the MTR28 mice relative to the wild type mice. Using different cDNAs as "driver" and "tester" two different libraries were produced. The MTR library was constructed using the cDNA derived from mammary glands from wild type mice as the "driver" selecting for genes up-regulated in the transgenic mammary gland. The WT library used cDNA generated from mammary glands from MTR28 transgenic mice as the "driver" selecting for genes down-regulated in the transgenic mammary glands. Approximately 1,440 clones from both libraries were sequenced by The Cincinnati Children's Hospital Research Foundation genome core facility. To date 576 clones from both libraries have been analyzed and approximately 40 percent of the sequenced clones were informative. Analysis of the sequencing results shows that genes involved in general cellular metabolism (13 genes) and fatty acid metabolism (11 genes) were the most affected by loss of TGF-β signaling. These result are similar to those described above for the Incyte gene chip. A complete overview of the categories of genes altered by loss of TGF-β signaling can be found in Table 1. Some of the genes identified in the libraries were shown previously to be regulated by TGF-β; for example, periostin (OSF-2) and selenoprotein P (12,13). Both libraries have been spotted onto glass slides at the University of Cincinnati microarray core facility creating the tissue specific gene chips. At the time of writing this report the libraries were being verified with cDNA generated from mammary glands isolated from transgenic and wild type mice treated with zinc sulfate for 1 week. Following verification with the mammary glands, the gene chips will be screened with cDNA produced from primary mammary stromal fibroblasts treated or untreated with TGF-β to identify genes directly regulated by TGF-β in the mammary stroma.

List of Key Accomplishments

Task 1. Induce Mammary Tumors in MT-DNIIR-28 (MTR-28) and Wild Type Mice, Months 1-24:

- Generation of mouse study groups
  - 26 Wild type mice with pituitary isografts and zinc-water ad lib.
  - 26 Wild type mice with pituitary isografts, DMBA treatment and zinc-water ad lib.
  - 27 MTR-28 transgenic mice with pituitary isograft, DMBA treatment and zinc-water ad lib.
  - 10 MTR-28 transgenic mice with pituitary isografts and zinc-water ad lib.
- Tumors generated in Wild type mice
  - X number of total tumors
  - Average of X numbers of tumors per mouse
- Tumors generated in MTR-28 Transgenic mice
  - X number of total tumors
  - Average of X number of tumors per mouse
- Histology of Tumors
  - Tumors derived from Wild type mice are adenoacanthomas
  - Tumors derived from Transgenic mice are adenoacanthoma and adenocarcinoma

Task 2. Produce and Screen Tissue Specific Microarray "Gene Chips", Months 18-36:

- Screened filter based and glass slide microarrys with RNA isolated from wild type and transgenic mammary glands and with RNA isolated from mammary fibroblasts treated or untreated with
  TGF-β
- Completed subtraction-suppression cDNA library construction
  - Subtraction was performed to select for genes up-regulated in transgenic mammary glands
  - Subtraction was performed to select for genes down-regulated in transgenic mammary glands
- Sequence analysis of clones
  - 624 clones have been sequenced
- Libraries have been arrayed onto glass slides by the Microarry facility at the University of Cincinnati
- Verification of a limited number of genes identified by filter based and glass slide microarray.
  - Northern analysis and Semi-quantitative RT-PCR were employed to verify gene expression.

List of Reportable Outcomes.

Abstract "Influence of Stromal Transforming Growth Factor-β Receptor Signaling on Mouse Mammary Neoplasia" presented at the 2002 Department of Defense Era of Hope Meeting in Orlando, FL Spetember 25-28.

References

1). Massague, J. 1998. TGF–β signal transduction. Ann. Rev. Biochem. 67:753-791.

2). Massague J. and Y.-G. Chen. 2000. Controlling TGF–β signaling. Genes and Dev. 14:627-644.

3). Moses, H. L., and R. Serra. 1996. Regulation of differentiation by TGF–β. Curr. Opin. Genet. Dev. 6:581- 586.

4). Pierce, D. F., M. D. Johnson, Y. Matsui, S. D. Robinson, L. I. Gold, A. F. Purchio, C. W. Daniel, B. L. M. Hogan and H. L. Moses. 1993. Inhibition of mammary duct development but not alveolar outgrowth during pregnancy in transgenic mice expressing active TGF–β1. Genes and Dev. 7:2308-2317.

5). Jhappan C, Geiser AG, Kordon EC, Bagheri D, Hennighausen L, Roberts AB, Smith GH, Merlino G. 1993. Targeting expression of a transforming growth factor beta 1 transgene to the pregnant mammary gland inhibits alveolar development and lactation. EMBO J. 12:1835-45

6). Gorska, A. E., H. Joseph, R. Derynck, H. L. Moses and R. Serra. 1998. Dominant-negative interference of the transforming growth factor–β type II receptor in mammary epithelium results in alveolar hyperplasia and differentiation in virgin mice. Cell Growth and Diff. 9:229-238.

7). Joseph, J., A. E. Gorska, P. Sohn, H. L. Moses and R. Serra. 1999. Overexpression of a kinase-deficient transforming growth factor–β type II receptor in mouse mammary stroma results in increased epithelial branching. Mol. Biol. Cell 10:1221-1234.

8). Matsui Y, Halter SA, Holt JT, Hogan BL, Coffey RJ. 1990. Development of mammary hyperplasia and neoplasia in MMTV-TGF alpha transgenic mice. Cell 61:1147-55

9). Pierce, D. F., A. E. Gorska, A. Chytil, K. S. Meise, D. L. Page, R. J. Coffey and H. L. Moses. 1995. Mammary tumor suppression by transforming growth factor β1 transgene expression. Proc. Natl. Acad. Sci. USA 92:4254-4258.

10). Bottenger, E. P., J. L. Jakubczak, D. C. Haines, K. Bagnall and L. M. Wakefield. 1997. Transgenic mice overexpressing a dominant-negative mutant type II transforming growth factor β receptor show enhanced tumorigenesis in the mammary gland and lung in response to the carcinogen 7,12-dimethylbenz-[a]-anthracene. Cancer Res. 57:5564-5570.

11). Barcellos-Hoff, M. H., and S. A. Ravani. 2000. Irradiated mammary gland stromal promotes the expression of tumorigenic potential by unirradiated epithelial cells. Cancer Res. 60:1254-1260.

12). Kruzynska-Frejtag A, Machnicki M, Rogers R, Markwald RR, Conway SJ. 2001. Periostin (an osteoblast-specific factor) is expressed within the embryonic mouse heart during valve formation. Mech Dev. 103:183-188

13). Mostert V, Wolff S, Dreher I, Kohrle J, Abel J. 2001. Identification of an element within the promoter of human selenoprotein P responsive to transforming growth factor-beta. Eur. J. Biochem. 268:6178-61 81.

Figure 1

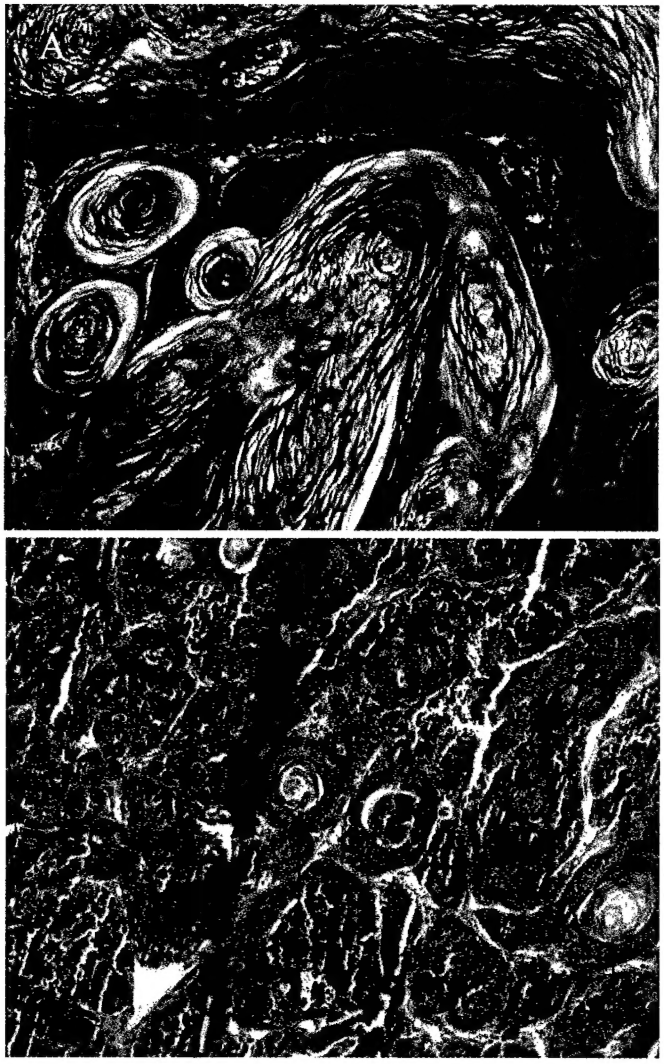

Figure 1. H and E staining of mammary tumors isolated from wild type mice. Wild type mice were given a pituitary under the kidney capsule at 5 weeks of age and placed on zinc-water at 6 weeks then treated with DMBA for four consecutive weeks. Mice were sacrificed when they appeared moribund or when the tumors reached an average of 2cm. A and B are two examples of tumors derived from the mice. Note the areas of swirling keratinization indicative of adenoacanthoma in A and B (arrows in B).

Figure 2

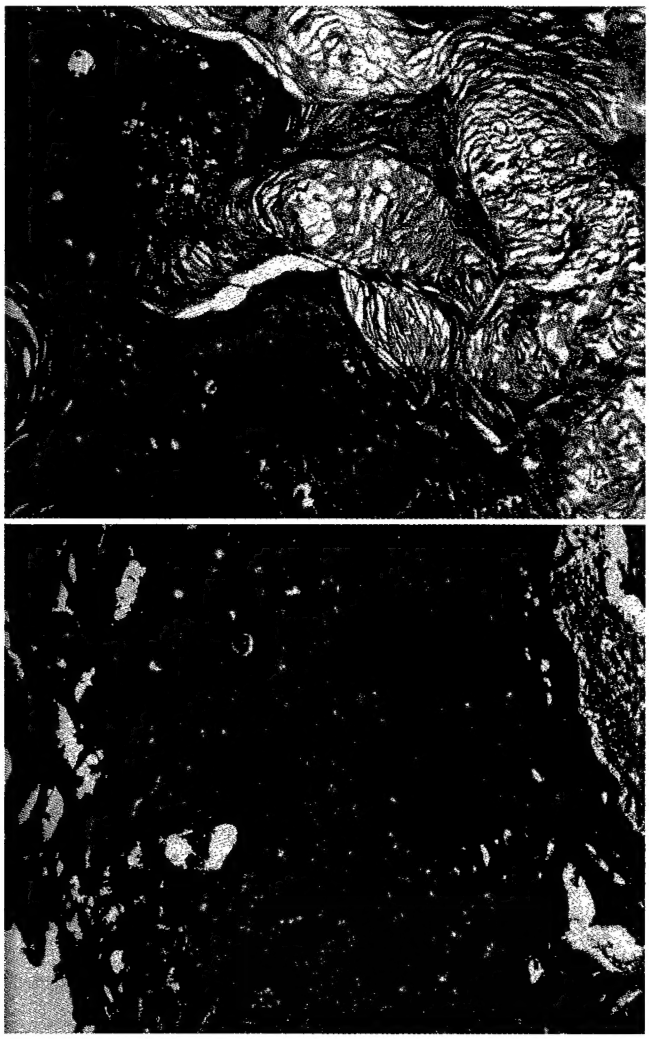

Figure 2. H and E staining of mammary tumors derived from MTR28 transgenic mice. The mice were treated as in the legend to Fig. 1 and the tumors removed from the mice when they reached an average of 2cm. A. Note the areas of keratinization (arrows) similar to the tumors isolated from the wild type mice. B. No areas of keratinization were seen in this tumor. There was a higher degree of eosin staining suggesting an increase in extracellular matrix deposition.

Figure 3

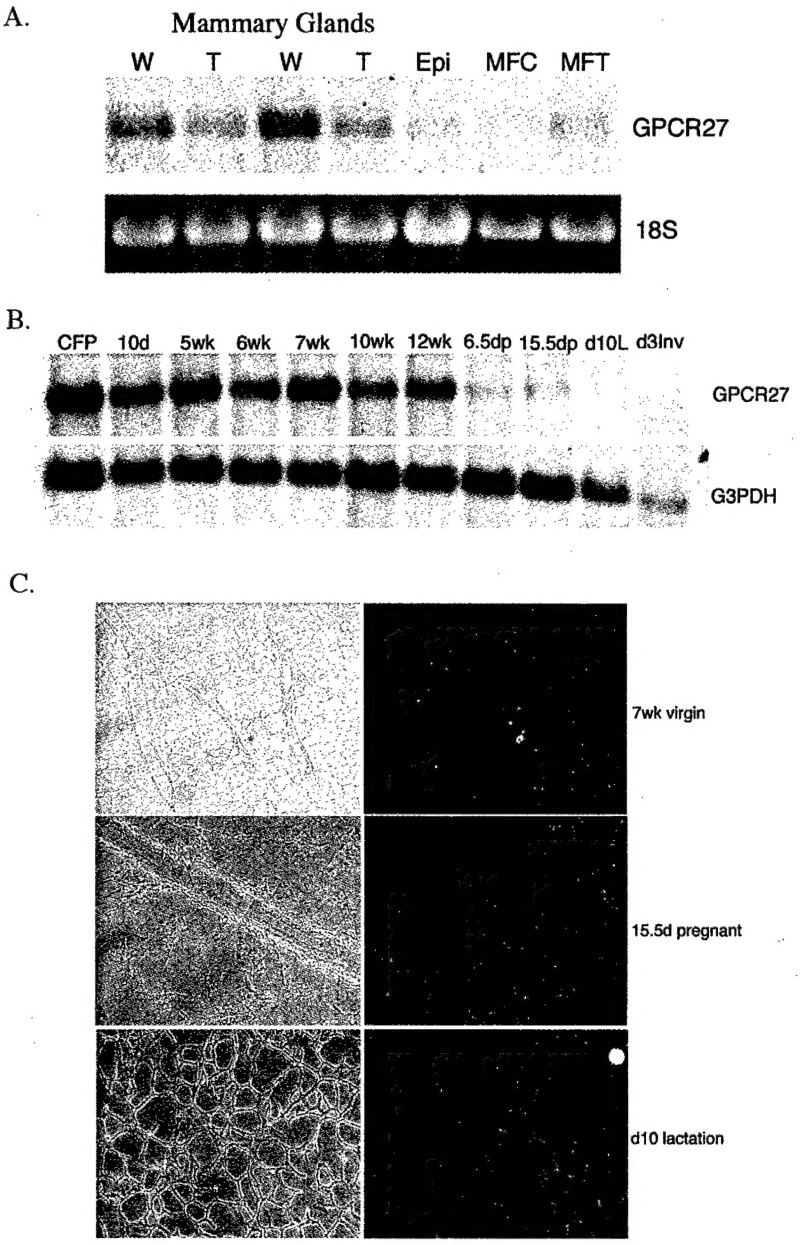

Figure 3. Verification of G-protein coupled receptor-27. A. Northern analysis of GPCR-27 expression in mammary glands from wild type (W) and Transgenic (T) mice and primary mammary epithelium (Epi) and mammary fibroblasts untreated (MFC) or treated (MFT) with 5ng/mL of TGF-β1. The 18S rRNA is shown for loading control. B. Northern analysis mammary glands at different stages of development including a cleared fat pad (CFP), postnatal day 10 (10d), glands from virgin animals from 5-12 weeks of age, early and late prognant animals (6.5dpc and 15.5dpc), day 10 of lactation (d10L) and 72 hrs of involution (d3Inv). Glyceraldehyde 3 phosphate dehydrogenase (G3PDH) was used as the loading control. C. Insitu analysis of mammary glands from wild type mice. The left panel shows the phase contrast image of the section. The right panel is the anti-sense with GPCR-27. The sense control gave no signal above background (not shown).

Figure 4.

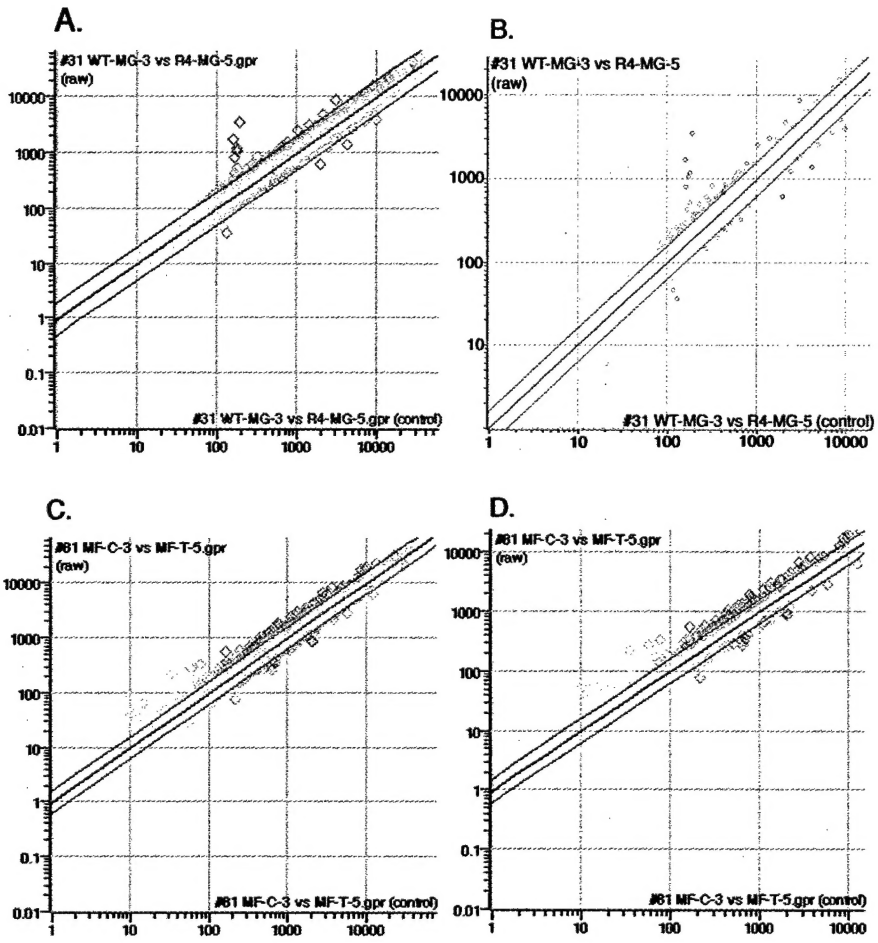

Figure 4. Scatter plot analysis for wild type vs. transgenic mammary glands on the Incyte gene array. RNA isolated from the mammary glands of 3 wild type (WT) mice was pooled as was the RNA from 3 transgenic (R4) mice to control for intersample variation (A and B). cDNA labeled with Cy3 and Cy5 was produced and hybridizd to the glass slides in triplicate. A. Results of the technical replicates of the data for the mammary glands. Normalization was done using LOWESS normalization, a global normalization accounting for intensity of the Cy3 and Cy5 labels on a given slide. B. The genes selected that were regulated 2 standard deviations from the mean. C and D. The same as A and B but with RNA isolated from mammary fibroblasts grown in culture either untreated (MF-C) or treated (MF-T) with 5ng/mL or TGF-β1.

Table 1. Genes regulated in MTR28 mammary glands relative to wild type.

Functional Category	Genes Up-Regulated	Genes Down-Regulated
Apoptosis	0	2
Cell Cycle	0	1
Cytoskeleton	2	4
ECM	4	3
Fatty Acid Metabolism	6	5
Immune Response	6	2
Lysosomal/Endocytosis	4	1
Metabolism	4	9
Proteases (non-lysosomal)	6	2
Protein Processing	4	2
Replication	1	0
RNA Processing	4	2
Signal Transduction	7	1
Transcription	1	0
Translation	2	0
Vesicle Trafficking	1	2
ESTs or Riken Clones	11	10